Structural changes of α-crystallin during heating and comparisons with other small heat shock proteins

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ABSTRACT

The small heat shock protein (sHSP) α-crystallin occurs in nearly all the major tissues of the body. It has two main functions; helping maintain transparency in the eye lens and as a molecular chaperone. We have investigated α-crystallin gels over a temperature range from 20 to 70ºC using wide and low angle X-ray scattering techniques. The low angle data show a moderate increase in both the spacing and intensity of the reflection from 20 to 45ºC. This was followed by a dramatic increase from 45 to 70ºC. Upon cooling, this effect was found to be irreversible over an eleven-hour period. Wide-angle scattering reflections from the α-crystallin gel arise from the secondary structure organisation, and can be characterized by inter-sheet (a ring at ~10 Å) and intra-sheet (a ring at 4.7 Å) interactions which appear to respond differently to increasing temperature. However, no indications of denaturation or unfolding are noticeable throughout the temperature range.

INTRODUCTION

The small heat shock protein (sHSP) α-crystallin occurs in nearly all the major tissues of the body. The highest concentration is to be found in the eye lens, where it occurs in very long fibre cells. It is the primary protein component and can approach 50% of the total dry weight of the lens [1]. One of its main functions here is the maintenance of short-range order in the lens cytoplasm, helping to achieve the refraction of light and to lens transparency in the visible spectrum [2]. Alpha-crystallin has two main isoforms, αA and αB, each with a molecular weight of about 20 kD. It is only in the lens that both isoforms are co-expressed. In other body tissues it is found at lower concentrations, with αB-crystallin by far the more prevalent [3,4]. In the lens however, the two isoforms co-aggregate into a heterogeneous population averaging a size of about 15 nm diameter and an overall mass of about 800 kD [5]. The α-crystallin subunit has unusual solution properties such that the aggregation process is propelled both by ionic and hydrophobic interactions. As a result the assemblages that are formed can vary considerably in number of subunits they contain. Recent studies of the aggregate structure of recombinant αB-crystallin using cryo-electron microscopy indicate that these assemblages share several structural features [6]. These include a spherical shell of about 3 nm thickness surrounding a hollow core of variable dimensions, and a dynamic region extending into the aqueous medium that appears to be quite flexible. The changeable structures of these assemblages has, so far, prevented crystal formation at the high concentrations of α-crystallin found in the lens *in vivo*. Thus the precise crystallographic structure of this protein is unknown. As α-crystallin is a member of the sHSP super-family, it exhibits chaperone-like activity and has been shown to prevent the superaggregation and/or precipitation of partially denatured target proteins at elevated temperatures [7]. Alpha crystallin is of great interest to medicine in general, as it is found at higher than normal concentrations in patients with ischemic heart [8]. It is also found at high levels in the brains of patients with Alzheimer's disease [9], multiple sclerosis [10] and other neurological diseases [11].

It has recently been shown that α-crystallin's chaperone-like activity at low and high temperatures is exhibited by very different populations of aggregates. At room and body temperatures, the size of the aggregate is typically around 15 nm. If the protein is heated, however, the aggregate size increases. A small gradual change in size can be observed between 20 and 50ºC, but by far the major changes occur above 50ºC, with aggregate size, shape, and mass fundamentally altered as indicated

by light scattering [12]; FPLC [13], non-denaturing gel electrophoresis, and transmission electron microscopy [14]. Such temperature-dependent structural changes are also observed for homopolymers of each α-crystallin isoform and for protein in the native isoform ratio that has been urea-denatured and then renatured [15]. There are also changes in the protein's secondary structure, as characterised by circular dichroism spectropolarimetry, which indicates alterations in β-sheet composition and/or stability in these samples after heating above 50ºC with subsequent cooling [14]. The mechanism by which this rearrangement occurs, and the organisation of sub-units in the resultant particles of each type remain the subject of much interest.

EXPERIMENTAL

α-crystallin was prepared from bovine calf lenses using a similar method of Thompson and Augusteyn (1983) [16], and concentrated by ultracentrifugation. Protein concentrations were determined spectrophotometrically, in serial dilutions using its extinction coefficient at 280 nm, or at 600 nm after reacting with Coomassie blue using bovine serum albumin as standard. The α-crystallin gel taken from the centrifuge precipitate was found to have a concentration of 296 mg/ml.

The α-crystallin gels were held in a temperature-controlled brass cell with a sample volume of 120µl, defined by a Teflon ring sandwiched by mica windows. For all specimens, 1min X-ray exposures were first taken at 20ºC. The temperature was then raised to 35ºC and left to equilibrate for a period of 10 minutes. The process was repeated in 5ºC steps until a temperature of 70ºC was reached.

The experiments were conducted on the low angle X-ray camera on station 2.1, and the wide angle diffraction station 14.1 at the Daresbury Synchrotron Radiation Source (SRS), UK. The X-ray patterns were recorded and analysed using BSL, OTOKO (Daresbury Laboratory, UK) and FIT2D [17] as well as Statistica (Statsoft, Tusla, USA) software packages. For most exposures, a vertical scan was taken through the centre of the pattern, resulting in a profile of the scattered intensity *I* versus radial position s. The intensity profiles were corrected for background scattering.

RESULTS

Low Angle Data

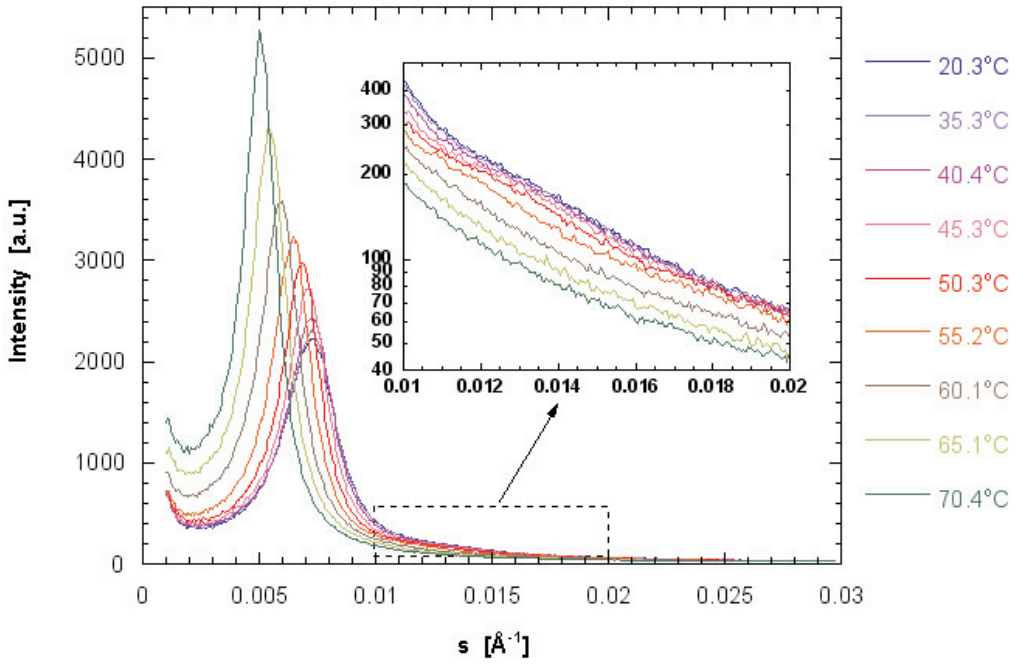
The scattering results for the α-crystallin gel (~300 mg/ml) are shown in Figure 1a). The X-ray intensity profiles are characterised by a strong peak, which with increasing temperature not only shifts its position to smaller scattering angles but also dramatically increases in intensity (by 67.3 % from 20ºC to 70ºC). The inset to Fig.1a) shows a weak secondary maximum , which disappears above 55ºC. At 20ºC the position of the main peak correlates with an average short-range distance between molecules of 138 Å. This result confirms the earlier work of Vérétout *et al* (1989) [18], who reported a broad reflection at room temperature of 14 nm at high concentrations, in the range about 100-300 mg ml^{-1}. The spacing of this reflection increases to 195 Å at 70ºC. This temperature-dependent change in spacing is highlighted in Fig.1b). The figure also emphasises that between 45ºC and 55ºC the gel undergoes a major transition, as both peak position and peak width change dramatically. Interestingly, both peak width and peak position correlate well above 55oC; however, in contrast to a fairly gradual increase in peak position between 20ºC and 50ºC, the corresponding peak width decreases steadily.

The sample measured at 70ºC was left overnight for a period of 11 hours to cool. Subsequently, a further X-ray scattering data set was taken at room temperature (19ºC) and compared with the curve measured at 70ºC (Fig.2). This shows that the effects of heating were essentially irreversible, as after cooling the peak position only increased a little correlating with a slightly lower spacing (i.e. from 195 Å at 70ºC to 192 Å at 19ºC). The intensity has increased at the peak position and decreased towards very low scattering angles, which indicates an improved X-ray scattering contrast. This change is most likely due to loss of water from the sample.

Wide Angle Data

The complementary wide-angle X-ray patterns of the native α-crystallin gel upon heating from 20ºC to 70ºC were also collected and studied. As the scattering is isotropic, the data were reduced to 1D scattering profiles in order to detect subtle changes (Figure 3). Within this scattering regime (0.075 $Å^{-1} \leq$

(a)



(b)

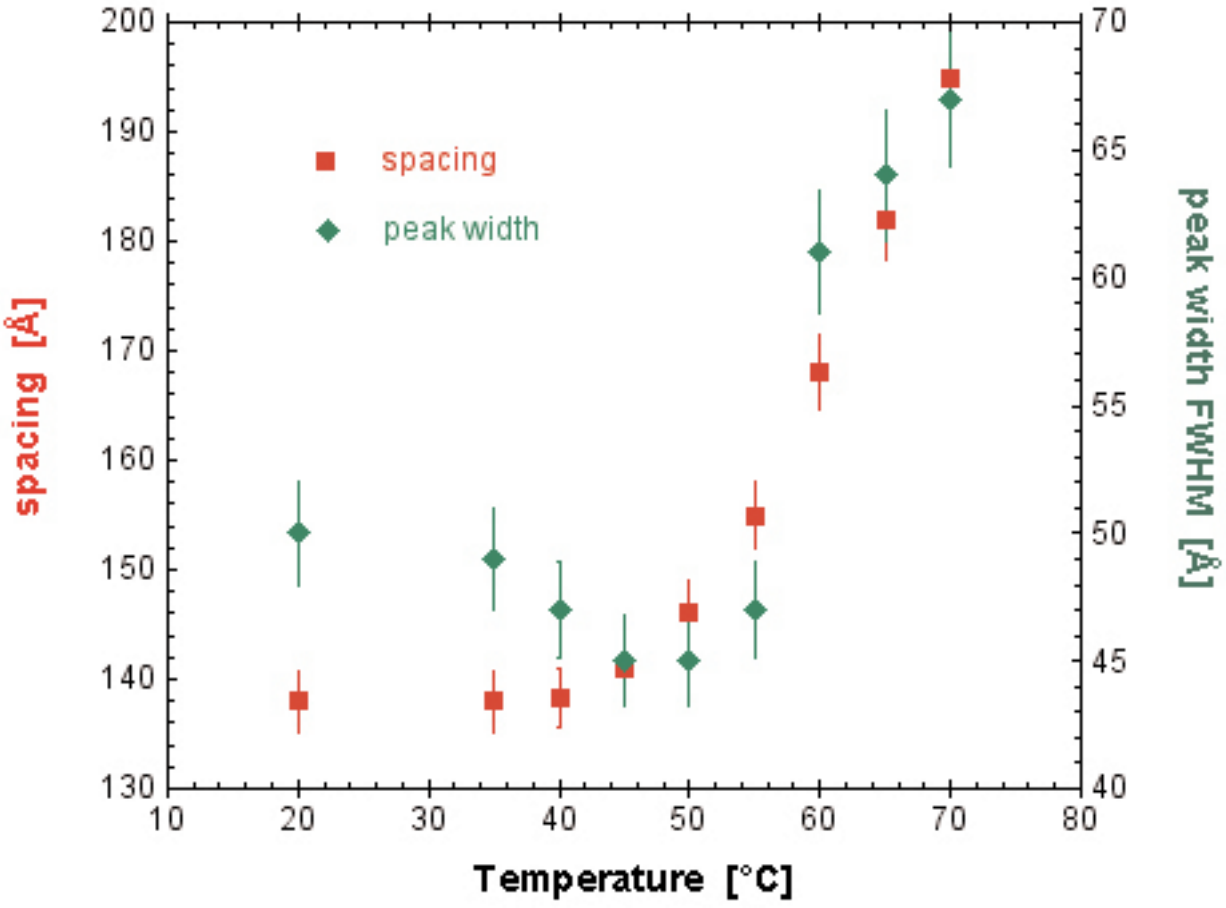


Figure 1 (a) X-ray intensity profiles of purified α-crystallin gel measured at temperatures between 20ºC and 70ºC showing the increase in both the spacing and intensity. The inset highlights the existence of a weak second-order peak at temperatures below 55ºC (note the use of a logarithmic scale). **(b)** The relationship between the position of the peak / the peak width (full length at half the maximum peak height) and increasing temperature.

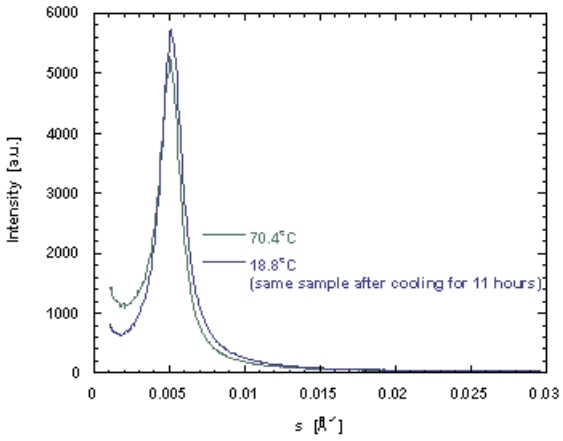


Figure 2 Comparison of intensity profiles measured for α-crystallin gel at 70ºC and after 11 hours when the sample was cooled down to room temperature (19ºC).

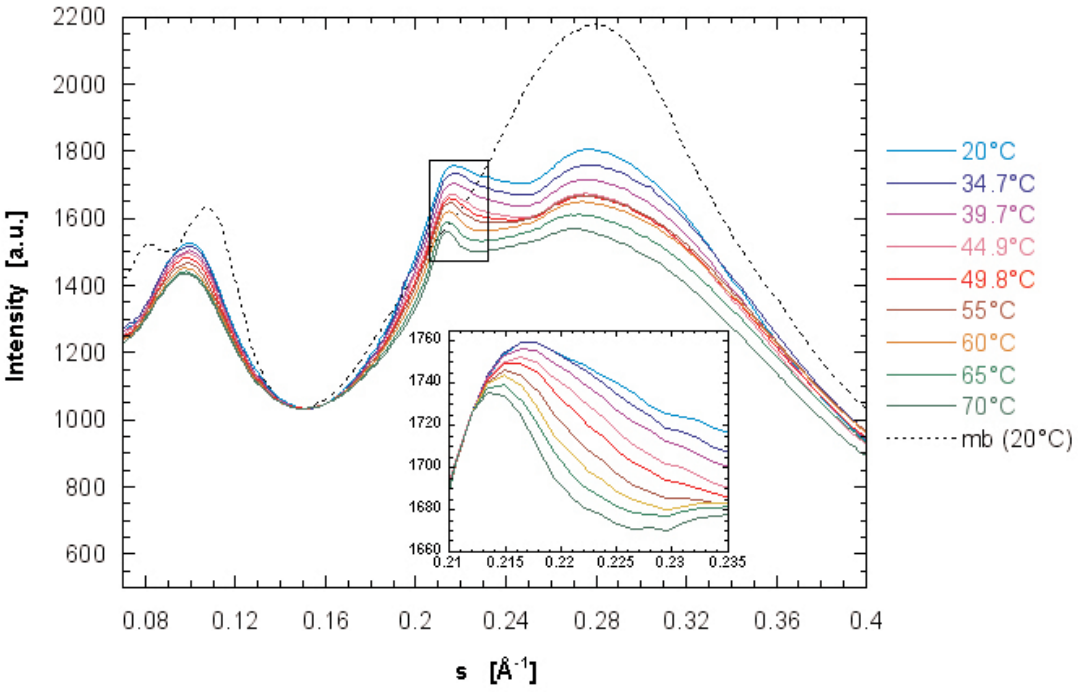


Figure 3 Reduced 1D wide-angle scattering data of native α-crystallin gel upon heating from 20ºC to 70ºC. The characteristic wide-angle features can be compared with a profile collected from a concentrated myglobin solution (~50mg/ml). Curves were normalized at s = 0.15 Å^{-1}. The insert shows the expanded region 0.21 Å^{-1} < s < 0.235 Å^{-1} highlighting the temperature-induced changes in the width of the peak corresponding to a Bragg spacing of 4.7 Å which is associated with the distance between β-strands. For the sake of clarity the curves in the inset were normalized at s = 0.212 Å^{-1}.

s ≤ 0.4 Å^{-1}) one can clearly see three major peaks around s = 0.1 Å^{-1}, s = 0.213 Å^{-1} and s = 0.275 Å^{-1}. The latter peak is relatively broad in comparison to the other two, and corresponds to a Bragg spacing of approximately 3.6 Å. It results from the diffuse scattering of water in the sample. However, the two other peaks, correlating to a Bragg spacing of 10 Å and 4.7 Å, respectively, are characteristic of β-sheets, and can be attributed to the high content of this secondary structure in α-crystallin. For comparison, Figure 3 also displays the wide angle scattering profile of a concentrated myglobin solution (i.e. a protein containing only helices as secondary structural elements) with peaks at around s = 0.083 Å^{-1} (12 Å) and s = 0.11 Å^{-1} (9 Å) corresponding to distances between helices. The 4.7 Å reflection in the α-crystallin pattern is diagnostic of the distance between neighbouring polypeptide chains in β-sheets (i.e. arises from the spacing between strands within a given sheet), while the 10 Å reflection is attributable to the separation of β-strands between sheets. It is the former peak in the isotropic wide-angle pattern of the α-crystallin gel that changes markedly during heating. With increasing temperature a reduction in

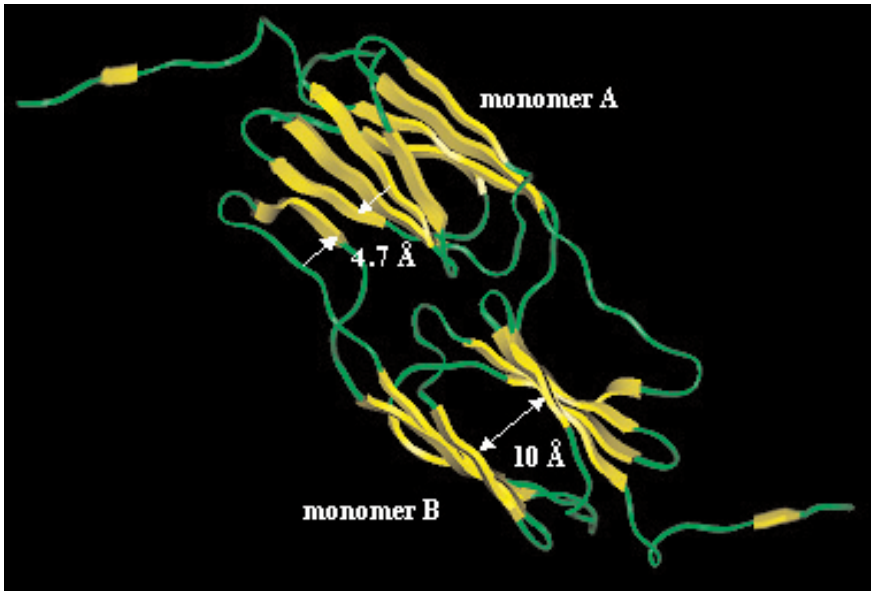


Figure 4. Ribbon model of a dimer taken from the structure of the 16.5kD small heat shock protein from *M. jannaschii* (pdb code 1shs), a structural relative of α-crystallin. Its secondary structure is dominated by β-strands (displayed in yellow) and the monomer-monomer interactions and dimensions of secondary structural units are shown.

peak width is clearly observable (see inset to Fig.3), indicating an increasing structural homogeneity and definition of this strand-strand interaction. This physical behaviour is in analogy to crystalline diffraction where a decrease in the peak width indicates an increase of the average size of perfectly crystalline regions. This improved structural uniformity of the strand-strand interaction at higher temperatures is in clear contrast to what one would expect if the protein had started to unfold or denature at elevated temperatures. Moreover, unlike the ring at 4.7 Å, the ring at around 10 Å remains effectively unchanged over the whole temperature range, indicating the conservation of the spacing between sheets with temperature.

DISCUSSION

The low angle studies of α-crystallin clearly show an increase in both the spacing and the scattered intensity. On increasing temperature the rise in scattered intensity indicates an increase in electron density of the aggregates. As the aggregates grow, so the average centre to centre distance between them also increases, which gives rise to the change in the spacing. Below 50°C, the rate of aggregate growth is low, while above 50°C, both the growth rate and the particle mass are higher. This is a striking phenomenon, which correlates well with an increase in functional efficiency that has been observed with increasing temperature for native α-crystallin [13, 19]. The presence of the secondary peak below 55°C (inset Figure 1) may indicate that below this temperature a weak long-range ordering of the α-crystallin molecules occurs in the gel state. Above this temperature the long-range ordering is lost completely due to thermal motion and the gel behaves similar to a dense liquid.

A focal point of the present study is the structural interpretation of the characteristic rings in the wide-angle scattering data. At protein concentrations as high as the concentration used here for the α-crystallin gel, the wide-angle scattering signal can provide valuable information on changes in the secondary structure. Over the temperature range considered herein, there is essentially no change in the spacing between the two β-sheets of the α-crystallin domain, nor is there a significant change in the spacing between β-strands. Nevertheless, the latter interaction becomes more uniform and homogeneous with increased temperature. These results differ from previous spectroscopic studies which show a loss of secondary structure with increasing temperature [13]. However we have to point out that our experiments were performed at much higher concentrations that are close to physiological conditions.

α-crystallin is a representative of the sHSP superfamily, varying in subunit size from 11-12 kD to 40 kD. They all share a common region which is referred to as the α-crystallin domain [20] spanning approximately 90-100 amino acids. The quaternary structure of sHSP superfamily members appears to be quite variable from one to another [21]. As

mentioned, α-crystallin aggregates are variable in size, shape, and mass, while other examples from the superfamily can show homogeneous aggregate sizes and symmetries. For instance sHSP 16.5 from *Methanococcus jannaschii*, assembles into a 24-subunit structure [22] which indicates that the region common to all superfamily members consists primarily of β-strands organized into two opposing sheets (see Figure 4). A loop within this region provides an additional short stretch of β-strand implicated in the formation of dimers, the basic structural unit for the aggregate. The C-terminal arms are involved in the formation of tetramers, with additional interactions leading eventually to the final closed-surface structure, while there is no structural information for the N-terminal region. In contrast, the wheat HSP 16.9 [23] exhibits very similar structural characteristics on the subunit level, but its quaternary structure (a dodecameric double disk) differs from the shell-type multimers seen in α-crystallin assemblies. This indicates that subunit interfaces and polypeptide extensions/deletions, which account for the high sequence variation in sHSP, correlate with the overall molecular shape of this protein family.

However, we would like to emphasize that recent studies [24-26] suggest that the exact dimer interactions of α-crystallin may differ greatly from other sHSP's. It has been shown that after deletion of the N-terminal domain of αB-crystallin no large oligomers are formed and that the C-terminal domain supports the assembly of a stable dimer which still retains chaperone-like activity [27]. Interestingly, it was observed by X-ray scattering that the αB-crystallin dimer in solution has a significantly different conformation compared to the dimer taken from the *M.jannaschii* sHSP crystal structure. Feil *et al.* [27] not only point out that this will result in different quaternary structures of the two proteins but also that the α-crystallin dimer interface is most likely very flexible. Given that the dimer appears to be a widespread building block in sHSP oligomer assembly [23], this flexibility is a crucial factor. It allows the overall quaternary structure of the α-crystallin oligomer to be variable and dynamic, since α-crystallins are shaped by a continuous exchange of subunits [28-30]. It is therefore conceivable that with rising temperature the α-crystallin aggregates become bigger as an increasing flexibility will facilitate association/dissociation events. Indeed Bova *et al.* [28] reported a 4.2 fold increase in the rate of subunit exchange by going from 37ºC to 42ºC. This dynamic structural behaviour would also explain the observed feature of the ring at 4.7 Å (associated with strand-strand interactions), which appears to be less well defined at temperatures below 50ºC. Considering physiological concentrations of around 300mg/ml and temperatures higher than 50ºC, the particle growth will eventually be limited by the interactions of relatively densely packed super-aggregates. As a result, subunit exchange reactions may become less frequent leading to a rather rigid and homogeneous shell structure. β-strand interactions (in particular those between neighbouring monomers) will become more stable and appear to be "locked" into position. This may explain why the width of the peak associated with a Bragg spacing of 4.7 Å becomes narrower at temperatures above 50ºC (Fig.3 inset). In contrast the 10 Å reflection, indicative of the distance between β-sheets, does not show any significant change over the temperature range. Consequently, it is tempting to suggest that this apparent change in secondary structure with heat is associated with the quaternary structural changes leading to larger and rather rigid aggregates which may be linked to a loss or decrease of chaperone-like activity above 50ºC.

In conclusion, we have shown that α-crystallin undergoes extensive structural changes with temperature with a major transition around 50ºC. Our findings confirm earlier observations at low concentrations of α-crystallin, which can now be confirmed at virtually physiological concentrations. The low angle results seem to apply both for low and high concentrations of α-crystallin. In contrast, investigating structural phenomena at almost physiological concentrations (as performed here on an α-crystallin gel) provided new insights into the secondary structure from wide-angle measurements. The study substantiates that α-crystallin function in the eye lens is closely associated with a highly dynamic oligomeric structure.

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